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MEASURING BACTERIAL LIPASE

by

David J. Lubert

Department of Dairying
UNIVERSITY OF ALBERTA

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MEASURING BACTERIAL LIPASE

by

David John Lubert, B.Sc.

A thesis submitted from the Department of Dairying in partial fulfillment of the requirements for the degree of Master of Science at the University of Alberta.

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MEASURING BACTERIAL LIPASE

INTRODUCTION

GENERAL

Of the types of spoilage occurring in dairy products one, resulting from enzyme action, is today receiving considerable attention. This type of spoilage, more commonly known as "rancidity", has been observed in milk, cream, butter, raw milk cheddar cheese, whole and skim milk powder and sweetened condensed milk. Rancid spoilage of cream during the winter months has been the cause of serious losses to the dairy industry in Alberta.

Investigations initiated at the University of Alberta in 1943 by the Departments of Animal Science and Dairying have attempted to determine the causes of rancidity in milk and the various factors affecting rancidity, so that ultimately measures could be recommended for the control and prevention of this defect (Dunkley et. al. 1946).

From the above investigations and those conducted elsewhere various facts are now available. It has been found, for instance, that rancidity in milk is brought about by enzymes which hydrolyze butterfat releasing fatty acids which impart to the milk objectionable odors and flavors. In addition to factors now known to inhibit and activate this hydrolysis of the butterfat, Castell (1942), it is also known that milk may become rancid with no apparent correlation between the rancidity and the numbers of bacteria present (Lubert, 1946).

This substantiates the work of those such as Herrington and Krukovsky (1939) who claim that rancidity in milk may be caused by a lipase of animal origin, i.e., secreted with the milk.

During the 1930's it was reported that butter manufactured from raw cream would become rancid if the cream contained sufficient numbers of certain microorganisms (Hammer and Collins, 1934). The most important organisms associated with such spoilage have been Pseudomonas fragi, Pseudomonas fluorescens and Achromobacter lipolyticum, all of which are lipolytic. These organisms have been isolated from air, water and improperly sterilized dairy plant equipment such as churns and pipelines.

When pasteurization of cream for buttermaking became popular rancidity caused by bacteria was much less widespread. Butter exhibiting the rancid defect when made from pasteurized cream has been so spoiled either by organisms surviving improper pasteurization or organisms gaining entrance to the butter during churning and subsequent handling of the butter. This holds true for butter made from cream not already exhibiting marked rancidity.

As a result of improved methods of production, neutralization, pasteurization and sanitation, bacterially-induced rancidity, especially in butter, has been reduced very considerably.

The incidence of hydrolytic rancidity in other dairy products follows a similar trend. However the mechanism of rancidity brought about by bacteria, with particular regard to cheddar cheese, is still not clearly understood.

In order to study rancidity by bacterial agents it is

necessary to be able to measure the lipolytic activity of a bacterial culture. This, of course, arises from the fact that the ability of an organism to break down fat governs its effectiveness in inducing rancid spoilage.

THE ACTION OF BACTERIA ON FAT

According to Frazier (1935) "The chief action of bacteria on fats is a hydrolytic cleavage by means of lipase, in which the fats are split into glycerine and fatty acids. Glycerine is readily acted on by a number of bacteria and is usually the first of the split products to be attacked. The course of the fermentation of glycerine will depend upon the organisms present and the general conditions but it is generally changed into lactic acid, volatile acids and aldehydes. The fatty acids which result from the hydrolysis of the fats or the fermentation of the glycerine are usually not attacked until the more available glycerine is destroyed. Then, however, these acids may be further split by some types of bacteria into simpler acids or to carbon-dioxide and water. The first part, then, of the action of bacteria on fat is usually hydrolysis and this is followed by oxidation processes."

Observations by Castell and Garrard (1941 b) appear to bear out the foregoing.

HISTORICAL

Various methods for the detection of lipolytic micro-organisms and studying lipolytic activity have been proposed during the past half century. In one of the earliest, the

natural fat technique, lipolysis is detected by a change in the appearance of small globules of fat distributed throughout a solid medium. Collins and Hammer (1934 a) have reported that Sommaruga, a pioneer in this field, in 1894 studied the lipolytic ability of microorganisms by growing them on solid media in which was dispersed olive-oil or other fat. The disappearance of the fat globules surrounding the growth of the bacteria was accepted as evidence of hydrolysis.

Eijkman (1901) poured melted agar over a thin layer of tallow in the bottom of a petri dish and found that the fat under lipolytic colonies became changed in color and opacity.

Anderson (1934) adapted the natural fat technique for use with a simple triglyceride. Adding neutral tributyrin to nutrient agar he used this turbid medium for the detection of lipolytic microorganisms. Conspicuous clear zones developed around lipolytic colonies.

Long and Hammer (1937) also modified the natural fat technique and obtained a more uniform fat distribution by adding natural fat in the form of an emulsion (fat-agar) to beef infusion agar. They observed that around actively lipolytic colonies the fat globules became more opaque. With the higher melting point fats which are opaque at room temperature they found it advantageous to warm the plates at 45°C prior to examination.

Limitations of the natural fat technique are that it often does not detect weakly lipolytic colonies and does not lend itself to quantitative interpretation.

Interest was also shown at the turn of the century in compounds which would impart to fat a particular color and to fatty acids a different color. This has been the basis of another method for the detection of lipase-producing micro-organisms. This method gained popularity when Smith (1908) observed that an aqueous solution of nile blue sulphate, when shaken with olein, stained the fat red but, when shaken with oleic acid, the fatty acid became blue or purple.

Turner (1929) added nile blue sulphate to an agar medium. To this, before solidification, he added a fat emulsion. A change in color of fat globules surrounding colonies from red to blue was taken as evidence of lipolysis. He observed that the dye was inhibitory to certain microorganisms.

Collins and Hammer (1934 a) (1934 b) using Hussong's (1932) modification of Turner's technique studied not only the action of certain bacteria on some simple triglycerides and natural fats, but also the types of lipolysis brought about by bacteria.

Stark and Scheib (1936) modified the nile blue sulphate technique by staining the fat and washing it free of excess dye before adding it to the agar. The inhibitory effect of the dye was therefore reduced.

Rosenfeld (1946) found that anaerobic lipolytic colonies on lipid agar containing neutral red base, were surrounded by a red areola while colonies themselves were extremely dark red. Non-lipolytic bacteria did not produce the color change from amber to red.

Other investigators such as Demeter (1938), Castell and Bryant (1939), Castell and Garrard (1941 b), Starr (1941), Knaysi (1941) and Richards (1945) have reported on the use of indicators other than nile blue sulphate for the detection of lipolytic microorganisms.

Castell and Bryant and Castell and Garrard (1941 a) substantiate the work of Jensen and Grettie (1937) by presenting evidence, resulting from the use of methylene blue and p-aminodimethyl aniline monohydrochloride, that bacteria may oxidize the fat in a medium. They concluded that the interpretation of color changes using nile blue sulphate was not a simple one. The presence of fat colored blue did not necessarily indicate hydrolysis alone.

A method for detecting microbial lipase was originated by Carnot and Mauban (1918) and later worked with by Berry (1933). Streaked agar plates, following incubation, were flooded with a saturated aqueous solution of copper sulphate. Bluish-green streaks of insoluble copper soap were accepted as evidence of lipolytic activity.

While the above methods are valuable for detecting lipolytic microorganisms they are of limited value for the quantitative determination of lipolysis.

Among the first to use a quantitative method for estimating bacterial lipase were Wells and Corper (1912). They introduced a definite amount of killed bacteria into various esters and neutralized the resulting emulsion with 0.1 N NaOH

to the end point of phenolphthalein. Further neutralizations were carried out at intervals during 14 days incubation. The final titration was made on an ether extract using alcoholic NaOH. Controls were heated prior to incubation and titration differences were used as measures of lipolysis.

Turner (1929) mentioned that lipase might be demonstrated in 24 hour autolyzed yeast peptone water cultures by titration with sodium alcoholate, with thymol as an antiseptic and tributyrin as the substrate.

The action of lipase was quantitatively measured by Trussell and Weed (1937) in cultures grown in beef infusion broth containing a fat emulsion. The cultures were titrated with 0.1 N NaOH, brom thymol blue being the indicator.

Castell and Garrard (1941 a) grew pure cultures of bacteria in a 50% mixture of triolein and 1% peptone solution for 17 days. Samples were withdrawn and titrated with 0.1 N NaOH in a neutral solution of equal parts of ether and alcohol, with phenolphthalein as the indicator. Distinct lipolytic action was concluded to be evident when the acidity (% oleic acid) was above 0.11.

Peters and Nelson (1947) (1948) added yeast in a buffered glucose broth to an emulsion of 5% butterfat in 0.25% agar (containing 1:1500 formaldehyde) and incubated the mixture for 48 hours. To an aliquot of the emulsion 25 ml of 95% neutral ethyl alcohol and 25 ml of diethyl ether were added, and the mixture titrated with 0.05 N KOH in methyl alcohol, using phenolphthalein as indicator. Titration difference

between the culture and a similar amount of the culture, boiled to inactivate the enzyme, was considered to indicate lipase activity.

In addition to the above procedures, Peterson, Johnson and Price (1943) have reported a method for the determination of milk lipase which is simple enough to be readily adapted to routine determinations. Skim milk was buffered at pH 8.5 using sodium diethyl barbiturate, tributyrin was added as a substrate, and after shaking, the reaction mixture was incubated at 40°C. Hydrolysis of the tributyrin was measured by removing aliquot portions of the reaction mixture at the beginning and at the end of the incubation period (15-120 mins.) and titrating them in the presence of alcohol and ether to the end point of thymolphthalein. This technique, however, involves the titration of a solution which contains proteins and, being buffered at pH 8.5, it has appreciable buffer capacity at the titration end point (approximately pH 10.2) and so does not give a sharp color change.

The necessity for titrating the liberated butyric acid in the presence of proteins and barbiturate buffer was eliminated in Dunkley's modification by acidifying the reaction mixture with phosphoric acid, extracting it with ether and titrating an aliquot of the ether extract.

PURPOSE

The following investigations were designed to show:

1. That the method as proposed by Dunkley and later modified by Smith (1949) could be applied to the determination of

the amount of lipolysis in skim milk cultures.

2. That the method could be used to study some of the characteristics of a bacteriallipase with particular reference to that produced by Pseudomonas fluorescens.
3. That the method could be applied to the determination of cheddar cheese lipase.

METHOD

Reagents

Borate Buffer - containing 6.23 g boric acid and 42 ml of N NaOH per liter.

Tributyrin - obtained from Eastman Kodak Company, Rochester, N.Y.

M H_3PO_4

Ethyl Ether

Titration Mixture - containing 1 liter of 95% ethyl alcohol, 1 liter distilled water and 50 ml of 0.1% alcoholic Cresol Red. A few drops of 0.05 N NaOH are added to give the mixture a pH of 7.4.

Standard 0.02 N NaOH

Procedure

The organism to be tested is inoculated by means of a loop from an actively growing 24 hour agar slant culture into 10 ml of sterile skim milk and incubated at its optimum growth temperature for 24 to 48 hours. (In preliminary work with this procedure larger amounts of medium were inoculated with smaller amounts of inoculum resulting in longer incubation times and lower titers.) Skim milk, unless otherwise noted, is skim milk which has been prepared from Bacto-Skim Milk (dehydrated).

Two ml of the resulting culture are pipetted into a test tube containing 5 ml of 0.1 M borate buffer and adjusted, if necessary, to pH 8.5 using M NaOH or M H_3PO_4. The buffered culture is placed in a water bath at 37°C and allowed to reach this temperature within 3 mins., 0.2 ml of tributyrin are added, a rubber stopper inserted in the tube and the

mixture shaken vigorously for 5 secs. to emulsify the tributyrin. The reaction mixture is returned to the water bath and is not agitated during the reaction period. At the end of the reaction period (30 mins. after the addition of tributyrin) 5 ml of M H_3PO_4 are added (to stop lipolysis and permit the subsequent extraction of the liberated butyric acid) and the whole mixed by inverting twice. The mixture is transferred to another water bath at approximately 20°C. After allowing a few minutes for the mixture to reach this temperature 10 ml of ether are added and the tube is shaken vigorously for 5 secs. The ether usually separates rapidly after this extraction, but almost 5 mins. should be allowed to permit small water droplets to settle out of the ether layer. The rubber stopper is loosened and after 3 mins. a 5 ml aliquot of the clear ether layer is transferred by means of a pipette to a 50 ml erlenmeyer or beaker containing 10 ml of titration mixture and titrated with 0.02 N NaOH to a faint orange color.

A blank or control determination is carried out in a similar manner except that the H_3PO_4 is added immediately after the mixture containing tributyrin has been shaken, i.e., the reaction period is omitted.

Hydrolysis of the substrate, referred to in this paper as lipolysis, is expressed as the difference between ml of 0.02 N NaOH required in the titration of the ether extract of the blank and the ml of 0.02 N NaOH required in the titration of the ether extract of the sample carried through the reaction period.

Titer, unless otherwise noted, is the average ml of 0.02 N NaOH required in duplicate titrations.

Titration differences (lipolysis) of less than 0.05 ml are arbitrarily interpreted as indicating no lipolysis, 0.05 - 0.1 ml as questionable lipolysis, 0.1 - 1.0 ml as weak lipolysis, and over 1.0 ml as strong lipolysis.

EXPERIMENTAL

A. FACTORS AFFECTING THE RESULTS

1. Extraction of Acids

The method described involves the titration of an ether extract of the acidified reaction mixture. All acids soluble in ether, if present, would contribute to the acidity of the extract. To determine the extent to which some acids soluble in ether were extracted by this method, solutions of acetic, propionic, butyric and lactic acid were first prepared and standardized as follows,

Acetic acid--------0.0192 N
Propionic acid-----0.0192 N
Butyric acid-------0.0197 N
Lactic acid--------0.0152 N.

Two ml of the acids were added to 7 ml of buffered fresh

skim milk in duplicate test tubes. In addition a 2.17 ml mixture of the 4 acids was added to a buffered sample of the skim milk. Tributyrin was added and, immediately after shaking, 3.5 ml of 1.5 M H_3PO_4 were added. The acidified reaction mixtures were then extracted with 10 ml of ether and aliquots were titrated with 0.0197 N NaOH.

Table 1 shows the percentage recovery of the various acids added to the buffered milk. Butyric acid was the most completely recovered, 90%, and lactic acid the least, 12%. The 46% recovery of the mixture of acids was in agreement with the theoretical 47% calculated from the percentage recovery of the individual acids. Duplicates checked within 2%.

Table 1

Recovery of Added Acids

Acid Added				
Kind	ml	Titer	Blank Corrected Titer	% Recovery of Acids
None	None	0.16	0.00	--
Acetic	2	0.39	0.23	23
Propionic	2	0.73	0.57	57
Butyric	2	1.06	0.90	90
Lactic	2	0.25	0.09	12
Mixture	2.17	0.62	0.46	46

Smith, using the above procedure, also found that 90% of added butyric acid was recovered in the ether extract and reported that there was a direct relation between the concen-

tration of free butyric acid in the sample and the amount extracted by the ether.

2. Source of Acid

(a) Acid Formation During the Reaction Period

Since the method measured acids soluble in ether, it was necessary to know if such acids were formed by buffered cultures during the reaction period in the absence of a substrate.

Cultures of Pseudomonas fluorescens and Escherichia coli were prepared as heretofore described. A series of buffered samples in duplicate were made up from these cultures and uninoculated skim milk and held over the reaction period. The buffered samples of E. coli were adjusted to approximately pH 8.5 using 5 N NaOH. The series was divided and to one half tributyrin was added, the other half being held over the reaction period without added tributyrin. The blanks were similarly treated.

Table 2

Acid Formation During the Reaction Period

Sample	ml Tributyrin Added	Blank Titer	Titer after Reaction Period	Lipolysis
Skim Milk	---	0.17	0.17	----
" "	0.2	0.20	0.20	----
P. fluorescens	---	0.17	0.17	----
" "	0.2	0.20	0.82	0.62
E. coli	---	0.69	0.69	----
" "	0.2	0.71	0.73	0.02

There was no increase in acidity of the ether extract of samples of uninoculated skim milk, samples of P. fluorescens, and samples of E. coli during the reaction period when no tributyrin was added (Table 2). Tributyrin increased the blank titers by 0.03 ml but no breakdown of tributyrin during the reaction period was evident in the uninoculated milk samples. The lipolytic value for E. coli of 0.02 ml was not significant, being within the range of experimental error. Acid formed during the growth of E. coli was accounted for in the blank. The addition of tributyrin to the P. fluorescens sample resulted in a high lipolytic value indicating the tributyrin had been partly broken down releasing butyric acid.

The above results seemed to justify the conclusion that an increase in titer at the end of the reaction period occurred only in the presence of tributyrin, providing the culture was capable of hydrolyzing the tributyrin.

(b) Cell Increase

Previously it was found that broth cultures of P. fluorescens grew well at 25°C but very slowly at 37°C. It was therefore assumed that there would be little increase in the number of cells during the reaction period. In support of this plate counts were carried out.

Two ml of a 96 hour skim milk culture of P. fluorescens (100 ml skim milk inoculated with 1 ml of a 48 hour skim milk culture and incubated at 25°C) were added to each of 6 sterile

test tubes. After the required amounts of borate buffer and tributyrin were added and the tubes shaken, 3 tubes were removed from the water bath. Two tubes were acidified, extracted and aliquots titrated. Dilutions of 1:100,000 and 1:1,000,000 were prepared from the third tube and plates were poured with T.G.E.M. agar (Standard Methods for the Examination of Dairy Products, 8th Edition, A.P.H.A. 1941). At the end of the reaction period the remaining 3 tubes were similarly treated.

All plates were incubated at 25°C for 72 hours and counted.

During the reaction period in which lipolysis took place to the extent of 0.33 ml no significant change in the number of cells was apparent (Table 3).

Table 3

Plate Counts and Lipolysis During Reaction Period

	Titer	Plate Counts /ml Dilutions	
		1:100,000	1:1,000,000
Before Reaction Period	0.24	4,200,000 4,100,000	7,300,000 7,300,000
After Reaction Period	0.57	4,300,000 4,700,000	6,300,000 7,600,000
Lipolysis	0.33		
Change in Plate Counts		+350,000	-500,000

If a number of cultures of different organisms had been so examined greater significance might be accorded to the above study in regard to the application of the method to a variety of organisms. Despite this limitation it was felt that, since there was no increase in the number of cells of P. fluorescens during the reaction period, change in the number of cells during the reaction period was eliminated as a possible source of ether-soluble acids.

(c) Protein as Acid Source

During the reaction period of the proposed method for the determination of lipolysis in skim milk cultures, the total acidity, in addition to the fatty acid produced, may be contributed to by amino acids resulting from increased protein breakdown. In studying this aspect of the problem changes in the amino N content during the reaction period were determined at the same time as the lipolysis was being measured. The amino N was measured using the Copper Method proposed by Albanese and Irby (1944).

Three drops of a 48 hour skim milk culture of P. fluorescens were used to inoculate an erlenmeyer flask containing 100 ml of sterile skim milk. The inoculated flask was incubated at 25°C for 48 hours.

Four 2 ml portions of the culture were aseptically transferred to test tubes and lipolysis determined. At the same time 20 ml of culture and 50 ml of the borate buffer were added to each of two 125 ml erlenmeyer flasks. When these had

reached 37°C in the water bath 2 ml of tributyrin were added to each and the mixtures shaken immediately. One flask was removed from the water bath and from it aliquot portions were taken for duplicate amino N determinations. The second flask was removed at the end of the reaction period and similar aliquots taken.

Table 4

Protein and Lactose Breakdown During the Reaction Period

	Titer	Amino N mg/2 ml of culture	Lactose g/2 ml of culture
Before Reaction Period	0.25	0.060	0.081
After Reaction Period	0.65	0.063	0.080
Lipolysis	0.40		
Change in Amino N and Lactose		+ 0.003	- 0.001

Figures for amino N and lactose content are the average of duplicates.

Lipolysis (Table 4) was observed to have taken place to the extent of 0.40 ml during the half-hour reaction period. During the same period, based on 2 ml of culture, an increase in amino N of 0.003 mg was noted. This increase was equivalent to 0.01 ml of 0.02 N NaOH. Subsequent observations on the increase in amino N during the reaction period have shown that it was approximately constant at 0.003 mg despite variations

in lipolysis.

Protein as a source of ether-soluble acid, contributing significantly to the titration increase of samples of P. fluorescens, was therefore eliminated.

(d) Lactose as Acid Source

Lactose breakdown during the reaction period also might be a source of ether-soluble acids measured by the method. Therefore, in conjunction with the amino N determinations, further aliquot samples were removed from the erlenmeyer flasks and lactose was measured using the gravimetric lactose method (Methods of Analysis, 6th Edition, A.O.A.C. 1945).

When lipolysis was 0.40 ml a decrease of 0.001 g of lactose occurred in the sample (Table 4). The 0.001 g of lactose, if converted completely to acids such as acetic and lactic, would contribute significantly to the value for lipolysis.

At this time the accuracy of this method of determining lactose was questioned on the grounds that it is actually a measure of the reducing capacity of a solution and unsuitable for measuring very small quantities of lactose.

In view of this and in view of the fact that previously E. coli, an organism capable of forming organic acids from lactose, did not produce an increase in acidity during the reaction period, it was assumed that lactose could be disregarded as affecting the values for lipolysis.

The conclusion that the increased titer obtained, at the end of the reaction period, with a sample of a culture of P.

fluorescens was due to acid released from tributyrin has been further substantiated by studies of other changes which might take place during the reaction period. Changes in number of cells, in the amino N and lactose content during the reaction period have been investigated and shown to be of little importance in affecting the results of lipolytic determinations.

3. Buffering at Various pH's

The effect of buffering at pH 5.5, 7.0 and 8.5 was investigated.

A buffer, designed to give a buffered sample of culture at pH 5.5, was prepared by adding 17 ml of M H_3PO_4 to 233 ml of M/15 $Na_2HPO_4.2H_2O$. A second buffer, designed to give a pH of 7.0, was prepared by adding 8.34 ml of 0.6 M H_3BO_3 and 0.25 ml of N NaOH to 41.41 ml of H_2O. The regular borate buffer was used to give samples buffered at pH 8.5.

Tubes of skim milk were inoculated in triplicate with P. fluorescens, Staphylococcus albus, E. coli, Bacillus subtilis and Alcaligenes faecalis. The P. fluorescens cultures were incubated at 21°C and the remaining cultures were incubated at 37°C for 72 hours. The 3 cultures of each organism provided samples for lipolytic measurements at pH 5.5, 7.0 and 8.5. Three 2 ml samples of each organism were buffered at each pH by adding to them 5 ml of the appropriate buffer. One of the 3 samples acted as a blank in each instance. All buffered samples were checked on a Beckman potentiometer and adjusted to their proper pH's where necessary.

At the lowest pH it was observed that the samples tended to coagulate when the buffer was added. The coagulum, which broke up during shaking, clung to the sides of the tubes after shaking. This could be a limiting factor in the application of this method at a low pH since the sample and occluded substrate clinging to the sides of the test tube would be at a different temperature during the reaction period than the buffered sample and substrate in the lower part of the tube. However the amount of material clinging to the sides of the test tube was small and the error so introduced was arbitrarily concluded to be small.

Table 5

Lipolysis at Different pH's

Organism	Lipolysis pH 5.5	pH 7.0	pH 8.5
Skim milk	---	---	0.02
P. fluorescens	1.01	1.37	2.28
S. albus	---	0.03	0.05
E. coli	---	0.05	0.04
B. subtilis	---	0.03	0.07
A. faecalis	0.03	0.06	0.07

Table 5 indicates that the lipolysis of cultures was measured by this method when samples were buffered at pH 5.5, 7.0 and 8.5. Although *P. fluorescens* gave evidence of

lipolysis when buffered at pH 5.5 and 7.0, maximum lipolysis was obtained at pH 8.5. All other cultures were non-lipolytic at pH 5.5, E. coli and A. faecalis were questionably lipolytic at pH 7.0 and S. albus, B. subtilis and A. faecalis were questionably lipolytic at pH 8.5.

The general effect of lowering the pH was one of inhibiting the lipolysis.

4. Concentration of Substrate

Smith in his investigations on milk lipase found that with highly lipolytic milks, using a 2 ml sample of milk, 0.2 ml of tributyrin as substrate were insufficient for maximum hydrolysis during the reaction period. To determine the effect of concentration of substrate on the measurment of lipolysis of a bacterial culture varying amounts of tributyrin were added to 2 cultures of P. fluorescens varying only in age.

Two erlenmeyers, each containing 100 ml of skim milk, were inoculated with 2 loopfuls of a 24 hour agar slant culture of P. fluorescens. Both were incubated at 27°C, one for 30 hours, the other for 48 hours. Subsequent treatment of each culture was identical.

Forty ml of culture were buffered with 100 ml of borate buffer and 7 ml of the buffered sample were pipetted into test tubes. Varying amounts of tributyrin were added to each when it had reached the reaction temperature. The remainder of the procedure was carried out as usual except that 3.5 ml of 1.5 M H_3PO_4 were used to stop lipolysis and 0.025 N NaOH was

used in the titrations. Blank determinations were also carried out using varying concentrations of tributyrin.

Table 6

Relation between Substrate Concentration and Lipolysis

Tributyrin ml	Lipolysis 30 hour Culture	Lipolysis 48 hour Culture
0.00	0.01	0.02
0.10	0.72	1.55
0.20	0.83	1.83
0.30	0.86	1.97
0.40	0.86	2.07
0.60	0.91	2.23
0.80	0.98	2.32
1.00	0.98	2.33
1.25	1.04	2.36
1.50	1.02	2.38
2.00	----	2.57

The results obtained for the two cultures of P. fluorescens are shown in Table 6. Curves A and B (Figure 1) represent the lipolytic activity of the 30 and 48 hour cultures respectively. Maximum lipolysis was obtained from the 30 hour culture when 0.2 ml of tributyrin were used but this amount of tributyrin was inadequate for the highly lipolytic 48 hour culture.

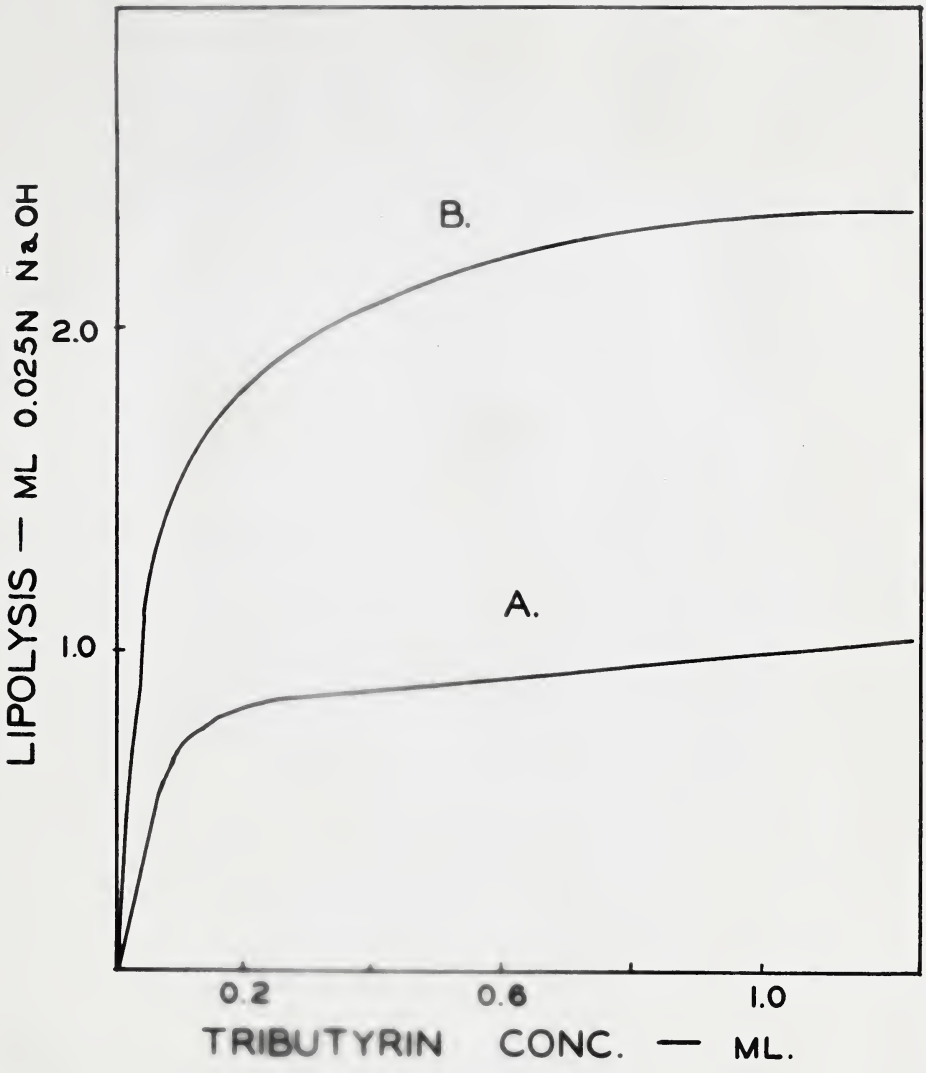

Figure 1 -- Relation between Substrate Concentration and Lipolysis

For cultures with lipolysis over 1.25 ml the amount of tributyrin should be increased to 0.8 ml. It is believed that for the great majority of cultures lipolysis will not be so high as to require an increase in the amount of tributyrin.

Smith has recommended that in the case of highly lipolytic milks the sample size might be reduced to 1 ml.

5. Variations in Replicate Cultures

Table 7

Variations in Replicate Cultures

Culture Number	Blank Titer	Titer after Reaction Period	Lipolysis
1	0.16	0.83	0.67
2	0.18	0.93	0.75
3	0.18	0.85	0.67
4	0.19	1.03	0.84
5	0.18	0.93	0.75
6	0.18	0.91	0.73
7	0.18	0.90	0.72
8	0.18	0.94	0.76
9	0.18	0.86	0.68
10	0.19	1.08	0.89
Milk Control	0.18	0.19	0.01

Ten replicate tubes containing 10 ml sterile skim milk were inoculated by means of a needle from a 24 hour agar slant

culture of P. fluorescens. The cultures were incubated at 21°C for 36 hours when the lipase activity of each was measured. For each replicate one 2 ml sample served as a blank and two 2 ml samples were held for the 30 min. reaction period. An uninoculated skim milk control was included in the series.

Lipolytic values for the 10 replicate cultures are shown in Table 7. The mean lipolysis was 0.75 ml. The highest and lowest values were found to deviate from the mean by approximately ± 13%. This deviation could be expected from growth differences between replicates.

6. Amount of Inoculum

To determine if the inoculum caused considerable variation in subsequent lipolysis by cultures, tubes of skim milk were inoculated by two methods as follows,

1. Three tubes were inoculated by a loop of an 18 hour agar slant culture of P. fluorescens.

2. Varying amounts (0.01 to 0.5 ml) of a suspension of P. fluorescens in sterile water were added to 12 tubes. The suspension was prepared by scraping the growth from an 18 hour agar slant culture into 5 ml of sterile water and mixing well.

All cultures were incubated at 21°C for 24 hours and lipolysis determined as usual except that one blank served for all tubes.

The three cultures inoculated by means of a loop gave lipolysis values of 0.28, 0.29 and 0.38 ml (Table 8). The amount of suspension and subsequent lipolysis did not bear an interpretable relation to each other although the larger

amounts of suspension, 0.20 and 0.50 ml were associated with the higher lipolytic values of 0.58 and 0.56 ml.

Since differences in lipolysis are likely to result from growth variations it is probable that loop inoculations are sufficiently accurate for most work with this method.

Table 8

Effect of Inoculum on Lipolysis

Method of Inoculation	Blank Titer	Titer after Reaction Period	Lipolysis
Loop	0.17	0.45	0.28
Loop	"	0.46	0.29
Loop	"	0.55	0.38
ml Suspension			
0.01	"	0.38	0.21
0.02	"	0.48	0.31
0.03	"	0.38	0.21
0.04	"	0.38	0.21
0.05	"	0.35	0.18
0.06	"	0.39	0.22
0.07	"	0.47	0.30
0.08	"	0.41	0.24
0.09	"	0.41	0.24
0.10	"	0.52	0.35
0.20	"	0.75	0.58
0.50	"	0.73	0.56
Milk Control	0.17	0.17	----

B. LIPOLYTIC ACTIVITY OF VARIOUS ORGANISMS

The method as previously described was used to compare the lipolytic activity of 11 species of 7 bacterial genera, an unidentified yeast and an unidentified mold. Included in the bacteria was an unidentified bacterium, isolated from rancid butter, which gives the odor of freshly cut apples.

Ten ml skim milk cultures were prepared and at the same time plates containing nile blue sulphate agar plus 0.5% tributyrin were inoculated with the bacteria with the exception of the yeast and the mold. All cultures were incubated for 72 hours at 25°C. The skim milk cultures were tested in duplicate for lipolysis by the proposed method and the giant colonies on the plates were examined for lipolysis as shown by blue stained globules surrounding the colonies.

Streptococcus lactis, Aerobacter aerogenes, Escherichia coli, Bacillus subtilis, Pseudomonas putrefaciens, Staphylococcus albus, unknown organism producing an apple odor, and a yeast proved to be non-lipolytic (Table 9). Alcaligenes faecalis, S. aureus and a mold were found to be questionably lipolytic and Mycobacterium smegmatis weakly lipolytic, while P. fluorescens exhibited strong lipolysis.

The observations of the action of the organisms on nile blue sulphate agar, containing tributyrin as substrate, are, for the most part, in agreement with the titration results. The qualitative, but incomplete quantitative, parallelism between the titration results and those of the nile blue technique may be accounted for by the following two observations:-

the bacteria was an unidentified bacterium, isolated from rancid butter, which gives the odor of freshly cut apples.

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1. The giant colonies on the nile blue sulphate agar were in contact with the substrate for a longer period than in the titration procedure and it was possible that oxidizing enzymes brought about changes which were interpreted as lipolysis.

2. The pH of the nile blue sulphate agar was approximately 7.0 while the cultures were buffered at pH 8.5 in the titration procedure and it is known that an enzyme that is active at one pH is not necessarily active at another pH.

Thornton, Shaw and Wood (1948) have shown that certain organisms are capable of producing a bitter defect in cream held at low temperatures. Six such organisms were supplied to the author.

Skim milk cultures of these organisms, identified as A1, 2 and 3, and B1, 2 and 3, were incubated at 27°C for 72 hours and their lipolytic activity measured.

Table 10

Lipolytic Activity of "Bitter" Organisms

"Bitter" Organism	Blank Titer	Titer After Reaction Period	Lipolysis
A1	0.16	0.28	0.12
A2	0.17	0.27	0.10
A3	0.16	0.33	0.17
B1	0.17	0.23	0.06
B2	0.15	0.22	0.07
B3	0.16	0.54	0.38

B3 proved to be the most lipolytic of the six "bitter" organisms (Table 10). By the previous criterion B1 and 2 and A2 were questionably lipolytic while A1 and 3 and B3 exhibited

weak lipolysis.

No correlation between the lipase activity and the bitter defect produced by the organisms was attempted.

C. LIPOLYSIS OF TRIBUTYRIN BY PSEUDOMONAS FLUORESCENS

1. Effect of pH During Reaction Period

Table 11

Composition of Sodium Borate Buffers used in Determination of Optimum pH for Hydrolysis of Tributyrin by P. fluorescens.

Buffer	ml of 0.6 M H_3BO_3	ml of N NaOH	ml of H_2O	Total volume ml
1	16.68	0.00	83.32	100.00
2	"	1.00	82.32	"
3	"	2.00	81.32	"
4	"	2.80	80.52	"
5	"	3.60	79.72	"
6	"	4.00	79.32	"
7	"	4.40	78.92	"
8	"	4.80	78.52	"
9	"	5.20	78.12	"
10	"	5.60	77.72	"
11	"	6.00	77.32	"
12	"	6.40	76.92	"
13	"	7.00	76.32	"
14	"	8.00	75.32	"
15	"	9.00	74.32	"
16	"	10.00	73.32	"
17	"	15.00	68.32	"
		ml of M H_3PO_4		
A	"	3.00	80.32	"
B	"	2.50	80.82	"
C	"	2.00	81.32	"
D	"	1.50	81.82	"
E	"	1.00	82.32	"
F	"	0.50	82.82	"

A series of borate buffers was prepared so that when 2 ml of culture were added to 5 ml of buffer a pH range from pH

4 to pH 11 was obtained. Each buffer was 0.1 M with respect to boric acid. The composition of these buffers is given in Table 11.

One hundred ml of sterile skim milk in an erlenmeyer flask were inoculated with 5 loopfuls of a 24 hour agar slant culture of P. fluorescens. The culture was incubated at 21°C for 48 hours.

Five ml of 12 buffers were pipetted into separate test tubes and 2 ml of the skim milk culture added to each. After warming to 30°C in a water bath and 0.2 ml of tributyrin had been mixed in each tube, the pH of each buffered sample was determined. A Beckman potentiometer, standardized at pH 8.60 and checked at pH 7.00 was used. The electrodes were rinsed with distilled water at 30°C and dried with paper tissue after each determination.

Lipase determinations were carried out as usual on duplicates of the above. Two blank determinations were carried out with culture buffered at pH 4.47 and 8.87.

A second culture, inoculated by means of 3 loopfuls of P. fluorescens from a 24 hour agar slant culture and incubated at 21°C for only 24 hours, was similarly treated. An additional buffer, buffering the culture at pH 9.67, was included in the case of this culture.

Table 12 and figures 2 and 3 show the results obtained for the 2 cultures of P. fluorescens. Figure 2 shows that at the beginning of the reaction period the optimum pH for the

48 hour culture was 8.87. Figure 3 shows the optimum pH for the 24 hour culture to be 8.92. On the basis of these results it is believed that cultures of P. fluorescens, when buffered at approximately pH 8.90, will give maximum lipolysis.

Table 12

Relation between Reaction pH and Lipolysis

Buffer	24 hour Culture pH	24 hour Culture Lipolysis	48 hour Culture pH	48 hour Culture Lipolysis
A	3.89	0.06	4.47	1.18
C	4.82	0.28	5.08	1.58
D	5.13	0.31	5.40	1.96
F	6.01	0.39	6.24	2.41
1	6.58	0.50	6.73	2.56
2	7.39	0.58	7.38	2.70
3	7.88	0.65	7.84	3.03
6	8.45	0.69	8.42	3.86
8	8.61	0.69	8.55	3.87
12	8.92	0.71	8.87	4.55
15	9.38	0.66	9.33	4.23
16	9.67	0.50	--	--
17	11.26	2.09	11.13	1.65

A markedly increased titer was obtained with a sample of the 24 hour culture buffered at pH 11.26. This was believed to be caused by saponification. Saponification, as a significant source of error in this method of determining lipolysis,

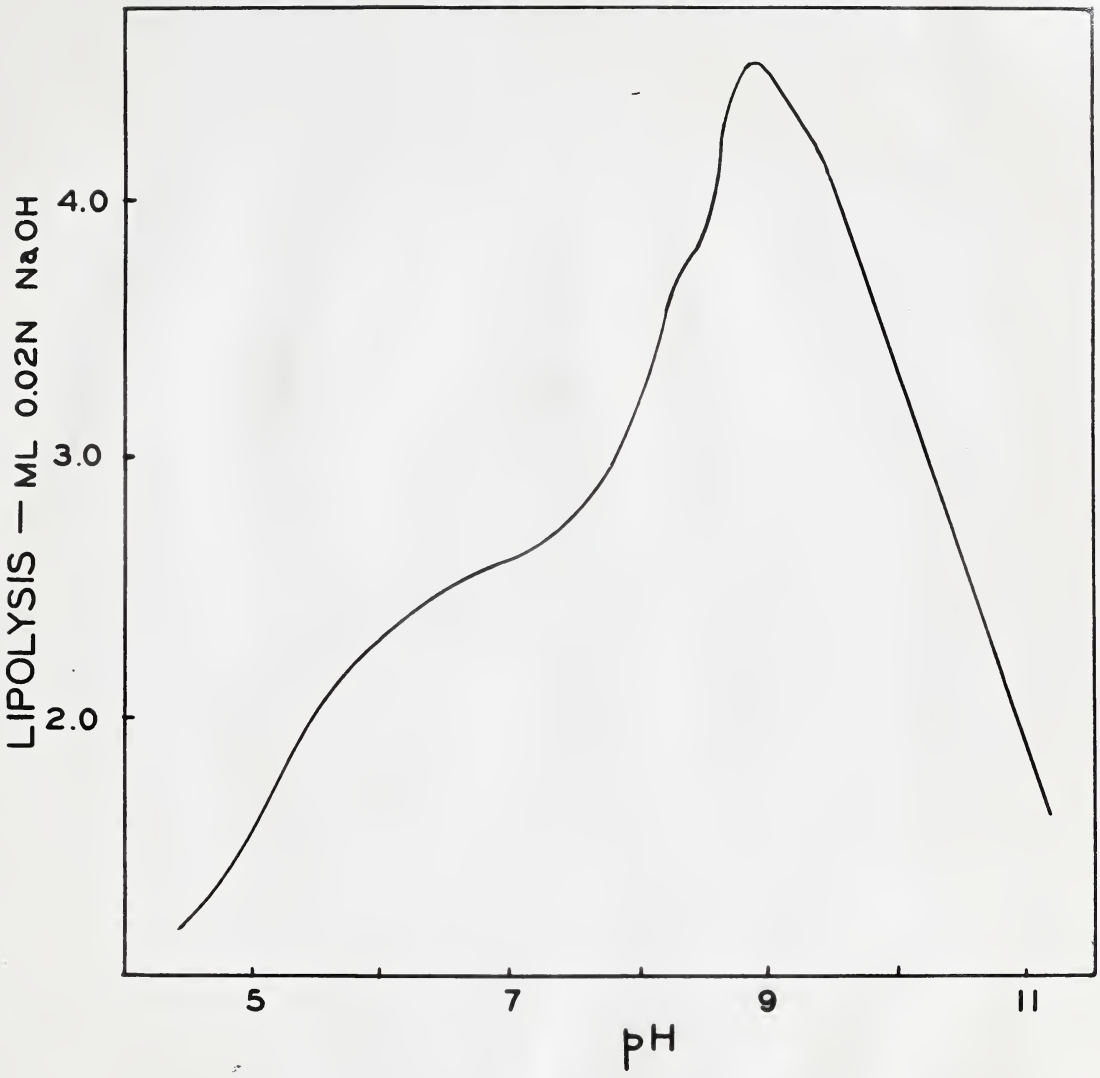

Figure 2 -- Relation between Reaction pH and Lipolysis

(48 hour culture)

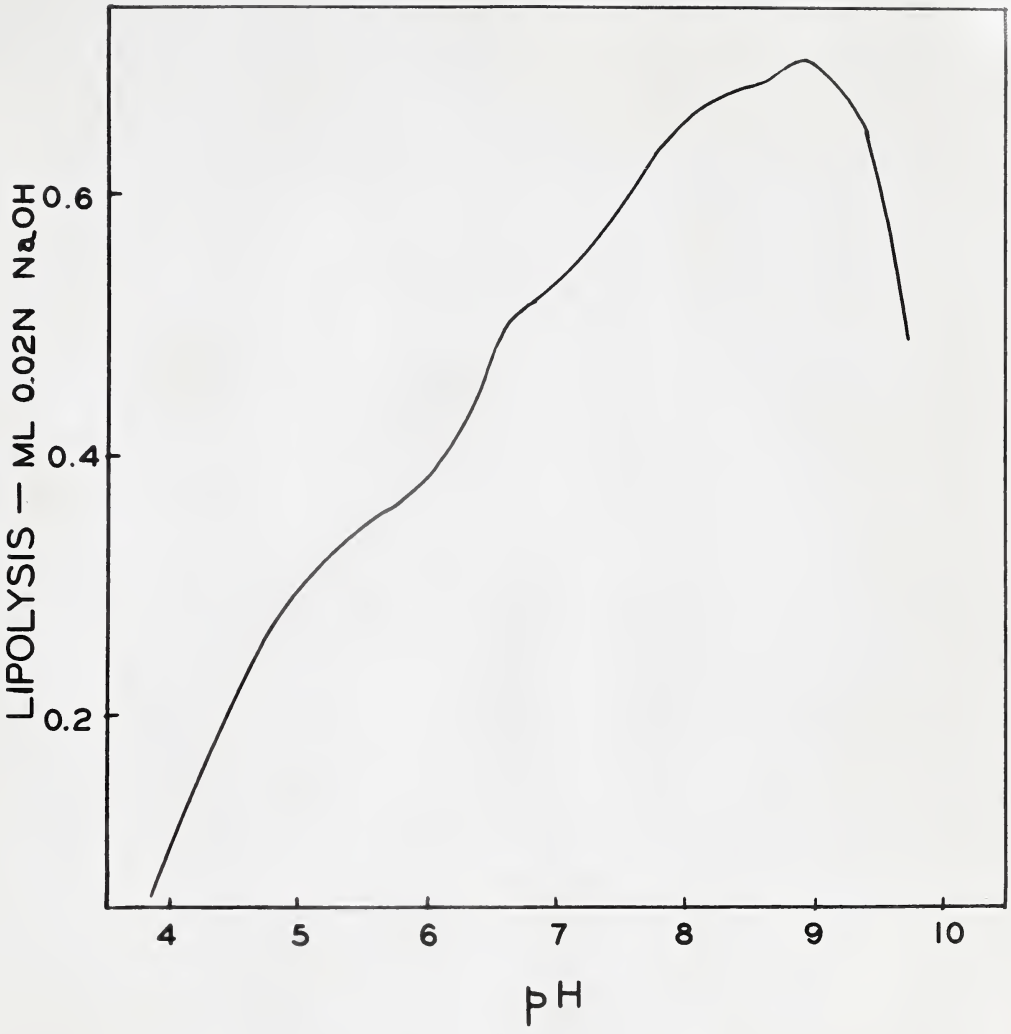

Figure 3 -- Relation between Reaction pH and Lipolysis
(24 hour culture)

was eliminated by Smith.

It is interesting to note that this pH optimum for P. fluorescens is almost the same as the pH optimum, 8.75, reported for milk lipase by Smith.

Quite apart from the effect of pH on hydrolysis it was found that culture buffered above pH 9.00 exhibited a marked greenish-yellow color. This would support the theory suggested by Young (1947) that pigment production is associated with alkalinity. Acid has been reported as destroying this color. In this study, when acid was added to old skim milk cultures of P. fluorescens showing strong greenish-yellow color, the color disappeared.

2. Effect of pH During Growth

Twenty-five ml of skim milk wereplaced in each of 12 large (20mm x 200mm) test tubes and sterilized. After cooling, varying amounts of sterile 5 M H_3PO_4 or sterile 5 N NaOH were added aseptically to the tubes and mixed well. Five ml of skim milk from each tube were removed aseptically and the pH determined at 22°C with a Beckman potentiometer previously standardized at pH 7.00 and checked at pH 4.00 and 8.60. Between each determination the electrodes were rinsed with distilled water and wiped dry with paper tissue.

All tubes were inoculated by means of a loop with P. fluorescens from a 24 hour agar slant culture. The cultures were incubated at 27°C for 24 hours. Following incubation 5 ml of culture were aseptically removed from each tube and the pH determined as above.

The preparation of the tubes and their resulting pH's are indicated, before inoculation and after incubation, in Table 13.

Table 13

pH Adjustment of Skim Milk

Tube	ml skim milk	ml 5 M H_3PO_4	ml 5 N NaOH	pH Before Inoculation	pH After Incubation
1	25	0.30	----	3.91	4.14
2	"	0.21	----	4.76	4.77
3	"	0.15	----	5.22	5.38
4	"	0.08	----	5.67	5.88
5	"	0.02	----	6.17	6.36
6	"	----	----	6.45	6.62
7	"	----	0.04	6.86	6.85
8	"	----	0.07	7.12	6.99
9	"	----	0.09	7.94	7.15
10	"	----	0.11	8.53	7.29
11	"	----	0.13	9.14	7.62
12	"	----	0.17	9.69	9.11

Duplicate 2 ml portions from each tube were pipetted into test tubes and buffered with 5 ml of the normal sodium borate buffer. The pH of the buffered samples was adjusted to approximately 8.5 using 5 N NaOH. Lipolysis was then determined in the usual manner with one buffered sample

serving as a blank in each set of duplicates.

Table 14

Relation between Growth pH and Lipolysis

pH Before Incubation	Blank Titer	Titer After Reaction Period	Lipolysis
3.91	0.12	0.12	----
4.76	0.12	0.12	----
5.22	0.13	0.90	0.77
5.67	0.15	1.16	1.01
6.17	0.16	1.95	1.79
6.45	0.18	2.16	1.98
6.86	0.17	2.17	2.00
7.12	0.18	1.87	1.69
7.94	0.19	1.97	1.78
8.53	0.21	2.05	1.84
9.14	0.20	1.12	0.92
9.69	0.12	0.14	0.02

Table 14 indicates the lipolysis obtained with P. fluorescens grown in skim milk adjusted to different pH values. P. fluorescens exhibited most lipolytic activity, 2.00 ml, after growth in skim milk adjusted to pH 6.86. No lipolysis was evident when the organism was grown in skim milk at pH 3.91, 4.76 or 9.69. Growth of the organism was undoubtedly inhibited in such milks. Good lipolysis took

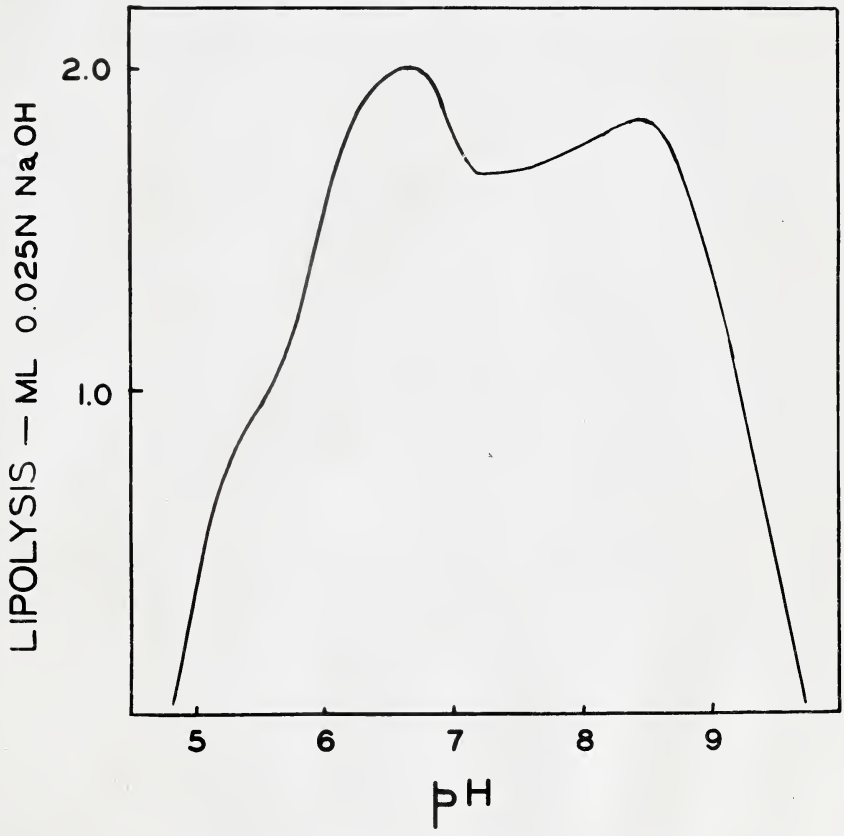

Figure 4 -- Relation between Growth pH and Lipolysis

place at pH 6.0 to 9.0 and a secondary optimum was found to exist at pH 8.53 (Figure 4).

There was little change in pH during the growth period when the organism was grown in skim milk adjusted to pH 6.86. The significance, however, of pH changes during the growth of P. fluorescens (Table 13) including the relation they may have to lipase production is not yet understood.

Although no criterion of growth was included in the above experiment the opinion of the writer is that maximum lipase production by P. fluorescens is associated with maximum growth of this organism.

In addition to pH observations it was noted that all samples which were lipolytic had an alkaloidal bitterness when tested organoleptically. This bitterness was most intense between pH 6.86 and 9.14.

3. Effect of Temperature During Reaction Period

Two ml samples of an 18 hour culture of P. fluorescens in skim milk were pipetted into a series of test tubes and buffered with 5 ml of the borate buffer. The buffered samples were then placed, in triplicate, in water baths adjusted to 6°, 19°, 26°, 37.5°, 41.5°, 46.5°, and 52°C and allowed 3 mins. in which to reach the temperature of the bath. One tube from each triplicate set served as a blank and the remaining 2 were held for exactly 30 mins. Lipolysis was measured in the normal manner (Table 15 A).

Subsequent to this an experiment of a similar nature was

carried out. It differed from the above in that a 24 hour culture of P. fluorescens, which had been held at 4.5°C for 1 week, was used and temperatures during the reaction period were 37°, 42°, 47°, and 51°C. Lipolysis is shown in Table 15 B.

Table 15 A

Relation between Reaction Temperature and Lipolysis

Temperature During Reaction Period °C	Blank Titer	Titer After Reaction Period	Lipolysis
6	0.16	0.34	0.18
19	0.17	0.65	0.48
26	0.17	0.90	0.73
37.5	0.16	1.35	1.19
41.5	0.16	1.37	1.21
46.5	0.17	1.33	1.16
52	0.17	0.38	0.21

Table 15 B

37	0.44	2.58	2.14
42	0.46	3.16	2.70
47	0.45	2.93	2.48
51	0.43	0.56	0.13

Curves A and B (Figure 5) indicate that maximum lipolysis was obtained when the reaction mixtures were held at 42°C during the 30 min. reaction period.

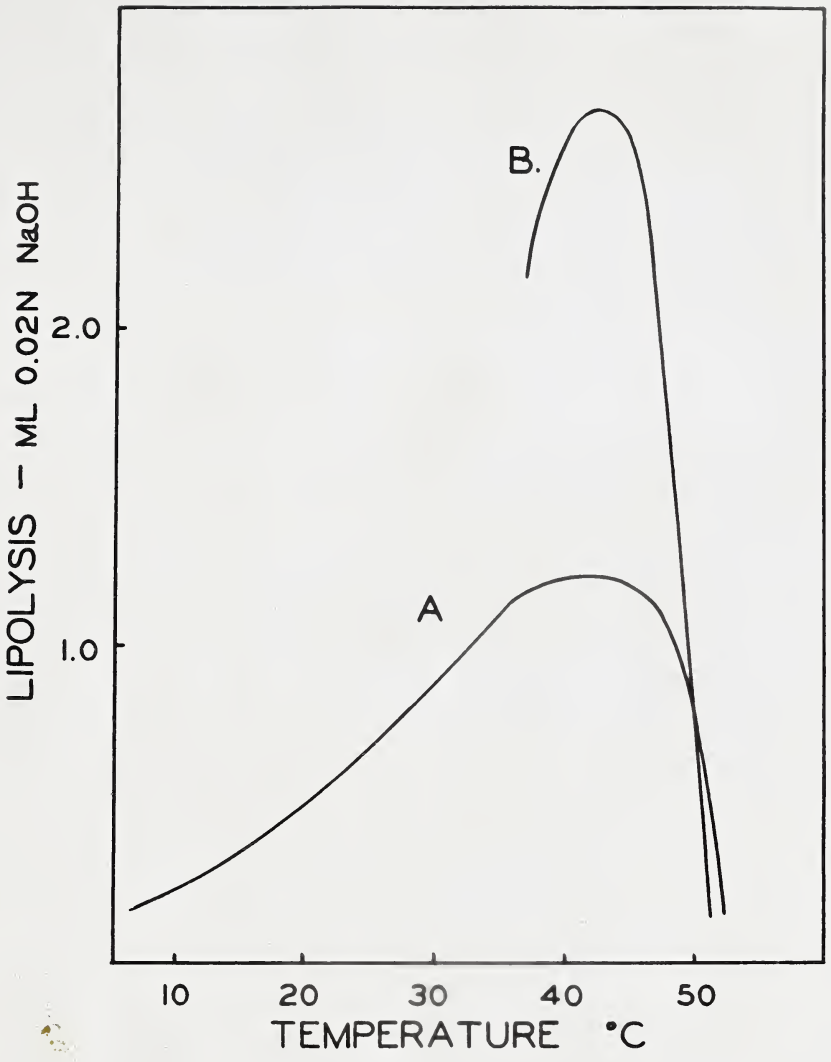

Figure 5 -- Relation between Reaction Temperature and Lipolysis

With the highly lipolytic 24 hour culture (Curve B) the temperature of the reaction period was more critical than with the less lipolytic 18 hour culture (Curve A).

During the reaction period heat has two effects on the lipolysis. Firstly, it supplies energy to the enzyme and below the optimum temperature this effect is dominant and the lipolysis is increased. Secondly, heat tends to denature the protein of the enzyme and above the optimum temperature and close to it this effect is dominant and the lipolysis is decreased. At the optimum temperature only a slight shift in conditions might be required to change the reaction over to one of inactivation. In view of this it was decided to retain a temperature of 37°C during the reaction period when measuring lipolysis by cultures of P. fluorescens.

Tables 15 suggest that the lipase of P. fluorescens would be almost completely inactivated during pasteurization. (i.e. 62°C for 30 mins.)

4. Effect of Temperature During Growth

Four skim milk cultures of P. fluorescens were prepared as usual and incubated at 4.5°, 21°, 27° and 37°C for 48 hours. The lipase activity of each was then measured using 37°C as the temperature for the reaction period and 0.025 N NaOH for the titration of the liberated acid.

Little or no lipase was produced when cultures of P. fluorescens were incubated for 48 hours at 4.5° and 37°C (Table 16). Optimum lipase production was obtained when the culture was incubated at 27°C.

Table 16

Relation between Growth Temperature and Lipolysis

Incubation Temperature of Culture °C.	Blank Titer	Titer After Reaction Period	Lipolysis
4.5	0.14	0.17	0.03
21	0.15	0.72	0.57
27	0.18	1.75	1.57
37	0.13	0.16	0.03

It is believed that long incubation periods (several days) at 4.5°C would result in measurable amounts of lipase being produced by P. fluorescens. However no experimental evidence is offered to substantiate this statement.

5. Effect of Various Media

Ten ml of nutrient broth, modified in various ways, and skim milk were inoculated with P. fluorescens in the normal manner. The broth media were modified as follows:

1. Broth + Oil -- 0.15% butter oil added to nutrient broth.
2. Broth + Agar - 0.05% agar " " " " .
3. Broth + Caseinate -- 0.05% sodium caseinate added to nutrient broth.
4. Carbohydrate-free broth -- Nutrient broth inoculated with E. coli and incubated 24 hours at 37°C. Culture

4. Continued -- filtered through a Berkefeld filter. Filtrate dispensed in 10 ml amounts and sterilized in the autoclave.

All cultures were incubated at 21°C for 24 hours and lipase determinations carried out with 1 sample of each culture as blank and 2 samples at the end of the reaction period. Buffered broth samples required adjustment with M H_3PO_4 to pH 8.5.

Table 17

Comparison of Media for Lipase Production

Culture Medium	Blank Titer	Titer after Reaction Period	Lipolysis
Broth + Oil	0.17	0.34	0.17
" " Agar	0.17	0.45	0.28
" " Caseinate	0.17	0.59	0.42
Carbohydrate-free Broth	0.19	0.46	0.27
Broth alone	0.22	0.53	0.31
" uninoculated	0.13	0.15	0.02
Skim milk alone	0.20	0.35	0.15
" " uninoculated	0.18	0.18	----

Of the media examined the least conducive to lipase production by P. fluorescens, proved to be skim milk, and nutrient

broth to which butter oil had been added (Table 17). Maximum lipase production took place when the organism was grown in nutrient broth containing sodium caseinate.

The use of nutrient broth containing either agar or sodium caseinate, as the culture medium prior to the measurement of lipolysis, is considered to be limited. Cultures in such media have been observed to be susceptible to the formation of gels when ether is added to the acidified reaction mixture and shaken. Gel formation does not allow a 5 ml aliquot of the clear ether extract to be obtained.

In preliminary investigations 0.7% peptone solutions were unsatisfactory for promoting the production of lipase. Jamieson's (1942) modification of *Sullivan's "K" Medium, asparagine in a buffered salt solution, was only slightly more effective than the peptone solutions. Fat-free skim milk (i.e. before reconstitution with water, the bacto skim milk was extracted with ether in a Soxhlet apparatus for 24 hours) was found to be equally as good a medium for lipase production as skim milk (i.e. Bacto skim milk without any special treatment).

Supplementing the data appearing in Table 17 a comparison of the lipase activity of 24, 48 and 96 hour cultures of P. fluorescens in skim milk, nutrient broth and carbohydrate-free nutrient broth is made in Table 18. Forty-eight hour cultures of P. fluorescens in nutrient broth and carbohydrate-

* Subsequently referred to in this paper as "K" medium.

free nutrient broth were more lipolytic than similar cultures in skim milk. It was felt that the tendency for gels to form with the former two media obviated their use in routine lipase determinations. The most reliable lipase results were obtained when the organism examined was incubated in skim milk despite the fact that various of the other culture media induced greater lipase production.

Table 18

Comparison of Media for Lipase Production

Culture Medium	Lipolysis 24 hr. Incubation	48 hr. Incubation	96 hr. Incubation
Skim Milk	0.29	0.86	2.63
Nutrient broth	0.57	1.25	2.13
Carbohydrate-free Broth	0.47	1.69	3.10

Since both agar and casein increase the lipolysis by P. fluorescens, it may be postulated that the colloidal state of the medium may be important in determining the amount of lipase produced. The presence of fat in the medium does not seem to be necessary for the production of this enzyme.

Since carbohydrate-free broth cultures were the most lipolytic, added weight is given to the statement inferring that the breakdown of carbohydrate in skim milk to organic acids was not a source of the acids extracted and measured by this method.

While the foregoing work with various media was being carried out, cultures of P. fluorescens in carbohydrate-free broth were observed to take on a marked green coloration. Such cultures when examined under ultra-violet light exhibited a pronounced light-green fluorescence. Nutrient broth and skim milk cultures of the same organism were only very slightly fluorescent. This particular property of carbohydrate-free broth was investigated further and is described under "Fluorescence"

6. Fluorescence

P. fluorescens was inoculated into "K" medium and variously modified nutrient broth. All test tubes contained 10 ml of the particular medium. Following incubation at 21°C for 72 hours the cultures were examined for extent of fluorescence by passing an ultra-violet light through the cultures. The examination for fluorescence took place in a dark room.

The media were prepared and inoculated as follows:

1. "K" medium --- Asparagine 1%
   K_2HPO_4 0.5%
   KH_2PO_4 0.5%
   $MgSO_4$ 0.2%
   Distilled water

   Inoculated with P. fluorescens as usual.
2. Nutrient broth inoculated with P. fluorescens as usual.
3. Nutrient broth inoculated with both P. fluorescens and E. coli.
4. Nutrient broth filtered through a Berkefeld filter, steri-

lized in an autoclave and inoculated with P. fluorescens as usual.

5. Nutrient broth filtered through a Berkefeld filter and inoculated with P. fluorescens as usual.
6. Nutrient broth inoculated with E. coli, incubated for 24 hours at 37°C, sterilized in an autoclave and inoculated with P. fluorescens as usual.
7. Nutrient broth inoculated with E. coli, incubated for 24 hours at 37°C, culture filtered through a Berkefeld filter, sterilized in an autoclave and inoculated with P. fluorescens as usual.
8. Uninoculated "K" medium.
9. Uninoculated nutrient broth.

Table 19

Fluorescence Production in Various Media

Medium	Fluorescence
1	Bright green ++++
2	Faint green +
3	" " +
4	" " +
5	" " +
6	" " +
7	Bright green +++
8	None
9	Light blue +

Table 19 shows that the culture of P. fluorescens in

"K" medium was the most fluorescent. The seventh medium, carbohydrate-free broth, exhibited good fluorescence. None of the several other modified broth cultures gave any evidence of increased fluorescence.

It appears that only the combination of growing E. coli in nutrient broth, filtering the resulting culture and sterilizing the filtrate in an autoclave resulted, in the above study, in a nutrient broth medium of increased fluorescence-producing properties for P. fluorescens.

If asparagine is unavailable for the preparation of "K" medium, the above-mentioned carbohydrate-free broth may be used in the examination of P. fluorescens for fluorescence.

Since cultures in "K" medium are poorly lipolytic it does not seem likely that lipolysis and fluorescence are related in any manner.

7. Effect of Calcium Chloride

It was found by Smith that 0.25 ml of 10% $CaCl_2$ when added to a 2 ml sample of milk increased hydrolysis as measured by the proposed method. The possibility that a similar effect might be obtained with cultures of P. fluorescens was investigated.

P. fluorescens was inoculated by means of a loop into 50 ml of sterile skim milk and the culture was incubated at 25°C for 72 hours.

To 4 of 6 test tubes 0.25 ml of 10% $CaCl_2$ was added. Buffer, culture and tributyrin were then added to the 6 tubes

in the normal manner. Two tubes were removed as blanks at the beginning of the reaction period. Lipolysis was measured without further deviation from the method as originally described.

The addition of 0.25 ml of 10% $CaCl_2$ to the reaction mixture to act as a lipase activator was not successful. The $CaCl_2$, as shown in Table 20, acted as a lipase inhibitor rather than an activator.

Table 20

Inhibition by Calcium Chloride

$CaCl_2$ Added ml	Blank Titer	Titer after Reaction Period	Lipolysis
0.25	0.32	2.79	2.47
----	0.32	3.34	3.02

8. Action on Different Substrates

Collins and Hammer (1934 a) have made the statement that, "When an organism hydrolyzed one fat it also hydrolyzed most others." In support of this statement the enzyme activity of a 48 hour culture of P. fluorescens in skim milk was determined using the proposed method with tributyrin, tricaproin and tricaprylin as substrates. The alkali used in the titrations was 0.025 N NaOH.

Table 21 shows that although tributyrin was hydrolyzed to the greatest extent of the three triglycerides, tricaproin and tricaprylin were also hydrolyzed appreciably. These re-

sults in a small way, substantiate the statement of Collins and Hammer.

Table 21

Action on 3 Triglycerides

Substrate	Blank Titer	Titer After Reaction Period	Lipolysis
Tributyrin	0.18	1.63	1.45
Tricaproin	0.56	0.98	0.42
Tricaprylin	0.27	0.51	0.24

This method of measuring lipase should be modified in some way to facilitate the addition of fats (as substrates) which are not in a liquid state. Fats composed of fatty acids insoluble in ether limit the usefulness of this method in carrying out investigations of the above nature.

9. Nature of the lipase

Growth of P. fluorescens on nile blue sulfate agar containing tributyrin is such that a clear halo appears surrounding the colony and extending out from the colony approximately 1 cm. In this clear area the globules of tributyrin have almost completely disappeared. It would appear that a fat-splitting enzyme capable of penetrating the medium for a considerable distance beyond the colony has been secreted by the organism. It is quite probable then, that a filtrate of a culture of this organism would possess some lipase activity.

A 48 hour litmus milk culture of P. fluorescens was filtered through a Berkefeld filter. The filtration of this culture proved very difficult and only a small amount of filtrate was obtained before the filter became so clogged that further filtration was impossible. Lipase determinations were, however, carried out on the filtrate obtained and the lipolytic activity of the filtrate was compared with that of the original culture.

The filtrate and original culture were lipolytic to the extent of 0.11 ml and 0.27 ml respectively. Because of the unsuitability of the filtration procedure little significance can be attached to these results. However they suggest that the filtrate possessed lipolytic properties and that the fat-splitting enzyme produced by P. fluorescens is an extracellular enzyme.

It is possible that with other methods of filtration more conclusive evidence might be obtained regarding the nature of the P. fluorescens lipase.

D. CHEESE LIPASE

In recent years rancidity in cheese has been associated with the production of lower fatty acids such as butyric acid by the action of milk lipase on milk fat. Peterson, Johnson and Price (1948 b) are of the opinion that milk lipase is inactive at the pH of cheddar cheese. Assuming this to be true, rancidity in cheese must be explained on the basis that other lipases are present in cheese. Peterson, Johnson and

Price believe that bacterial lipases are partially responsible for the more rapid flavor development in raw milk cheddar cheese as compared to cheddar cheese made from identical milk after pasteurization.

In arriving at the conclusion that the milk lipase plays no part in the flavor development of cheddar cheese these authors have used data concerning the lipase activity of milk and cheese. The method used for determining lipase in cheese was essentially the same as the Peterson, Johnson and Price method of determining milk lipase.

A cheese extract was prepared first by mascerating 20 g of cheese in 100 ml of water in a Waring Blendor, passing it through a hand homogenizer and centrifuging at 2000 r.p.m. A portion of the supernatant was buffered with 2 ml of 0.38 M aniline buffer at pH 5.0 and diluted to 12 ml with water. Tributyrin (0.2 ml) was added and shaken. A 5 ml aliquot was removed immediately for titration while the remainder was incubated for 60 mins. at 40°C after which a further aliquot was removed and titrated. Titration differences between aliquots represented tributyrin hydrolysis. The titration was carried out by adding the aliquot to 5 ml of 0.02% alcoholic thymolphthalein. Two ml of ether were then added and the solution titrated to a definite blue with 0.1 N alcoholic NaOH.

It was felt that confirmation of the results of Peterson, Johnson and Price and further information regarding cheese could be gained by applying the method of determining bacterial lipase, as herein proposed, to cheese extracts.

Extracts of 4 samples of commercial raw milk cheddar cheese, varying from 15 days to 91 days old, were prepared according to the method of Peterson, Johnson and Price (1948 a). Duplicate 2 ml portions of each extract were buffered with 5 ml of a phosphate buffer (8.75 ml M H_3PO_4 and 1 ml M NaOH were added to 117 ml of M/15 $Na_2HPO_4.2H_2O$) and adjusted to approximately pH 5.0 with M H_3PO_4. The remainder of the procedure was carried out as usual except that a 45 min. reaction period was used.

Table 22

Lipase Activity of Cheese Extracts

Cheese Sample	Age of Sample Days	Blank Titer	Titer After Reaction Period	Lipolysis
1	15	0.21	0.24	0.03
2	27	0.19	0.23	0.04
3	54	0.20	0.24	0.04
4	91	0.25	0.25	----

No cheeses examined exhibited lipolysis (Table 22). An extract of a "Blue" cheese at a later date also had no lipase activity at pH 5.0.

These results could not be reconciled with the fact that Peterson, Johnson and Price had stated that, "Most of the lipase active at pH 5.0 is set free between the fifth and hundredth day of ripening." However, as the result of

a personal communication with Mr. M. H. Peterson it was learned that some commercial cheddar cheeses exhibit no lipase activity at pH 5.0 at all and that some parts of a cheese may be entirely lacking in lipolytic activity.

To counteract the latter, he recommends the use of extremely large samples or several small samples (20 g) with subsequent averaging of the lipolytic activities of these samples.

If most cheddar cheeses contain lipases active at pH 5.0 it is difficult to understand why more of them do not become rancid. It may be that a lipase whose optimum activity is at an alkaline pH but having some activity at a low pH (e.g. P. fluorescens lipase) is important in causing cheese to become rancid.

DISCUSSION

The term lipase has been used exclusively in this paper although in most cases the activity being measured was that of a more specific enzyme, tributyrinase. Since the proposed method does, however, measure the activity of other triglyceride-splitting enzymes the use of the term lipase, referring to enzymes whose major specificity is toward glyceride substrates, was felt to be justified.

It has been suggested that the first action of bacteria on fat is a hydrolytic cleavage by means of lipase into glycerol and fatty acids, this being followed by oxidative processes. Measurements of the lipolytic activity of bacteria, then, are actually concerned with the hydrolytic cleavage. If the oxidative processes are given time to act they will complicate the interpretation of lipolytic results. By allowing only a short time during which the bacterial enzymes may act on the fat the complicating oxidations may be materially reduced and possibly eliminated. The relatively short period (30 mins.), of the method heretofore described, in which the bacterial enzymes may act, make it superior, in the opinion of the writer, to methods in which the bacteria are in contact with the fat for many hours.

The 30 minute reaction period may, however, be criticized since weakly lipolytic cultures may not have sufficient time in which to produce a measurable effect on the fat. Longer incubation periods of such cultures prior to testing for lipolysis may prove advisable.

This may partially explain the results indicating that cultures of Staphylococcus were questionably or weakly lipolytic while Trussell and Weed have reported members of the genus Staphylococcus to be markedly lipolytic.

Enzyme preparations used throughout this investigation have been crude and no attempt has been made to purify them. Such purification might obviate the routine use of the method.

Indications are that the lipase of P. fluorescens will act in varying degrees on tributyrin when exposed to a variety of conditions. For example, fairly extreme conditions of pH and temperature are necessary to inactivate the lipase completely. While experimental evidence is lacking this may be pertinent to the rancid spoilage of dairy products.

Although information regarding a greater diversity of microorganisms would materially augment this study it is believed that sufficient data have been presented to recommend the use of the method in the study of lipolysis of microorganisms. It is hoped that such studies will eventually aid in a better understanding of the significance of bacterial lipolysis in dairy products.

SUMMARY AND CONCLUSIONS

1. Another method of determining lipolysis by microorganisms has been presented.

2. Experimental data have been offered to show that the method quantitatively measures the acid released from the substrate during the reaction period and that some other possible sources of acid, such as protein and lactose, do not affect the results. Although samples of a P. fluorescens culture buffered at pH's 7.0 and 8.5 gave higher results, lipolysis was also evident at pH 5.5. A substrate concentration of 0.2 ml tributyrin was shown to be adequate when lipolysis was below 1.0 ml. Variations in lipolysis between replicate samples of ± 13% of the mean were shown to exist and inoculation by means of a loop was concluded to be satisfactory for most work with this method.

3. Measurements with the proposed method using an arbitrary criterion have shown cultures of S. lactis, A. aerogenes, E. coli, B. subtilis, P. putrefaciens, S. albus, an unknown organism producing an apple odor and a yeast to be non-lipolytic. A. faecalis, S. aureus and a mold were questionably lipolytic and M. smegatis weakly lipolytic while P. fluorescens exhibited strong lipolysis. Of 6 "bitter" organisms similarly compared 3 were weakly lipolytic and 3 were questionably lipolytic.

4. The lipolysis of tributyrin by P. fluorescens has been studied and results indicate that:-

A. Skim milk, as a culture medium, was the most reliable

for lipolysis determinations. Production of the enzyme was best when the organism was grown in skim milk adjusted to a pH nearly neutral and when the culture was incubated at 27°C.

B. Good lipolysis occurred when the reaction mixture, i.e., the medium in which the substrate was dispersed, was buffered at a pH of approximately 8.90 and the reaction temperature was 37°C.

C. Since tricaproin and tricaprylin were also broken down the lipase was not specific for tributyrin.

D. The evidence that the fat-splitting enzyme produced by P. fluorescens is extracellular was indicative, but insufficiently strong to permit a definite conclusion.

E. In the concentration used calcium chloride acted as an inhibitor to lipolysis.

5. Good fluorescence was obtained when P. fluorescens was grown in carbohydrate-free broth.

6. Extracts of raw milk cheddar cheeses have been prepared and examined for lipolytic activity using the proposed method with a reaction period of 45 mins. None of the extracts so examined exhibited lipolysis.

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